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The genetic control of flower–pollinator specificity

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The ca. 275,000 species of flowering plants are the result of a recent adaptive radiation driven largely by the coevolution between plants and their animal pollinators. Identification of genes and mutations responsible for floral trait variation underlying pollinator specificity is crucial to understanding how pollinator shifts occur between closely related species. *Petunia*, *Mimulus*, and *Antirrhinum* have provided a high standard of experimental evidence to establish causal links from genes to floral traits to pollinator responses. In all three systems, MYB transcription factors seem to play a prominent role in the diversification of pollinator-associated floral traits.

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Introduction

Most flowering plants rely on animal pollination for reproductive success. Flower–pollinator interactions are considered to be a major driver for floral trait diversification and angiosperm speciation [1–3]. A key observation supporting this proposition is that many angiosperm species produce flowers with a particular pollination syndrome, a suite of floral phenotypes that enable specialized associations with the 'attraction and utilization of a specific group of animals as pollinators' [2]. These traits include flower size, color, scent, texture, shape, orientation, reward (e.g. nectar, pollen, fragrance), and pistil and stamen arrangements. For example, hummingbird-pollinated species typically have red flowers with a floral tube, copious nectar production, and exserted stamens and pistils, whereas bee-pollinated flowers display various colors (but usually not red), a smaller quantity of nectar, inserted stamens and pistils, and a clear landing platform. A molecular description of the genetic control of such pollinator specificity is crucial to understanding how pollinator shifts occur between closely related species, which are often associated with dramatic floral trait divergence and pollinator-mediated reproductive isolation and speciation.

Since the early 1990s, quantitative trait locus (QTL) analysis of floral traits that affect pollinator preference has been carried out in a few plant lineages such as *Mimulus* [4], *Aquilegia* [5], *Petunia* [6], and *Iris* [7]. These studies suggest that many pollinator-associated floral traits are controlled by few loci with large effects. However, progress in identifying the specific genes and mutations that are responsible has been quite slow until recently. This is perhaps not surprising because most plant systems for investigating flower–pollinator interactions were not particularly amenable to fine-scale genetic analysis, especially before the advent of massively parallel sequencing technologies.

Another factor that has impeded a deep understanding of the genetic control of flower–pollinator interactions is the admixture of different standards of evidence that have been used in the literature to link genotype to phenotype to pollinator response. Overall, correlative evidence is prevalent in linking genes to floral phenotypes, and pollinator responses to a particular floral trait are often assumed instead of being tested in controlled experiments. Here we first attempt to lay out a common set of experimental evidence that is necessary to establish a causal link from gene to floral trait to pollinator response, and then discuss recent studies that best fit these criteria.

The evidence necessary to link genotype to phenotype to pollinator response

Ideally, a causal link between genotype and phenotype can be established by a combination of fine-scale genetic mapping and functional characterization through transgenic manipulations. We consider that a genotype–phenotype link is formally established if at least one of the two following requirements is fulfilled: (i) fine-scale genetic mapping to the single gene level; (ii) QTL mapping or co-segregation analysis indicates a candidate gene and transgenic manipulations of the candidate gene result in expected phenotypes. Neither of these two lines of evidence has been readily available for most plant systems that are used to study flower–pollinator interactions. However, whole genome sequencing is becoming a routine practice — even for non-model systems — which makes fine-scale genetic mapping feasible, and the development of transformation protocols for a non-model plant is tedious but not always difficult.

Once the genetic basis of a floral trait is determined, pollinator foraging assays (in controlled artificial

environments or natural habitats) are required to determine how pollinators respond to alternative phenotypes produced by the different alleles using carefully constructed plant materials. Transgenic lines with manipulation of a single gene would be ideal to test the role of this gene in pollinator preference with absolute confidence. When dealing with the effect of loss-of-function alleles, induced recessive mutants that differ from the wild-type parental line only at the target locus could be equally appropriate. If these materials are not available, a third (suboptimal) alternative is near-isogenic lines (NILs) that differ from the parental line only in a small region of the genome that contains the causal gene, although in this case precautions should be taken to ensure the substituted genomic region does not affect other pollinator-associated floral traits, especially less immediately obvious traits such as scent or texture.

In recent years considerable efforts have been made to identify genes underlying pollinator-associated floral trait variation in several plant systems, including *Petunia*, *Mimulus*, *Antirrhinum*, *Ipomoea*, *Clarkia*, and *Phlox*. For example, in *Ipomoea*, down-regulation of the *flavonoid 3′-hydroxylase* (*F3′H*) gene because of *cis*-regulatory change in some species resulted in flower color change from blue/purple to red [8], which is correlated with the transition from bee-pollination to hummingbird-pollination. In *Clarkia*, up-regulation of the *S-LINALOOL SYNTHASE* (*LIS*) gene contributes to the strong scent emission in *C. breweri*, the only moth-pollinated species in the genus [9]. However, in neither of the two systems has pollinator response to allelic variants of the identified genes been tested, leaving the significance of these individual genes in controlling pollinator preference unresolved. In *Phlox*, *cis*-regulatory changes in the *flavonoid 3′5′-hydroxylase* (*F3′5′H*) gene and an *R2R3-MYB* transcription factor gene have been implicated in flower color change leading to pollinator-mediated speciation by reinforcement [10,11]. However, direct evidence from fine-scale mapping or transgenic manipulations to verify the gene identity is still lacking, and the pollinator-mediated selection in this case is because of pollinator constancy rather than the pollinator specificity that is required for a shift between pollinator guilds [11]. It is the other three systems (i.e. *Petunia*, *Mimulus*, and *Antirrhinum*) that have contributed the most rigorous experimental evidence to our current knowledge of the genetic control of flower–pollinator specificity, and these will be discussed in more detail.

Petunia — from flower color to scent

Petunia integrifolia, *P. axillaris*, and *P. exserta* are closely related species displaying a typical bee, hawkmoth, and hummingbird pollination syndrome, respectively [12]. *Petunia integrifolia* has purple, scentless flowers with a short, wide corolla tube and little nectar; *P. axillaris* has white, fragrant flowers with a long, narrow corolla tube and a large volume of nectar; and *P. exserta* flowers are bright red, scentless, with exserted stamens and pistils and copious nectar (Figure 1).

A key regulator that controls the flower color difference between *P. integrifolia* and *P. axillaris* was identified as *ANTHOCYANIN2* (*AN2*) [13,14••], encoding an R2R3-MYB transcription factor. A typical flowering plant genome harbors >200 *MYB* genes, ~2/3 of which encode transcription factors with two adjacent MYB domains (i.e. R2R3-MYBs) and ~1/3 with a single MYB repeat [15–17]. AN2 belongs to subgroup 6 of R2R3-MYBs [15,17] that form a protein complex with basic helix–loop–helix (bHLH) and WD repeat proteins to activate anthocyanin biosynthesis in most anthocyanin-pigmented flowers, including *P. integrifolia* [13,14••,18]. The white color of *P. axillaris* results from loss of AN2 function through multiple independent acquisitions of nonsense or frameshift mutations in the *AN2* coding DNA regions [13,14••].

To investigate how alternative *AN2* alleles impact pollinator preference, Hoballah *et al.* [14••] transformed the functional *P. integrifolia AN2* allele into the *P. axillaris* background, converting the white flower to purple, while all other floral traits remained the same as in wild-type *P. axillaris*. When tested in controlled greenhouse conditions, hawkmoths showed marked preference for the wild-type white flower over the purple transgenic flowers, whereas bumblebees showed preference in the opposite direction. The *AN2* case thus fits our criteria for linking genotype, phenotype, and pollinator response by taking original genetic data from hybrid crosses and transposon tagging, verifying and characterizing the effects of the locus with transgenic plants, and then using those plants to assess pollinator response in a controlled greenhouse environment [13,14••].

The genetic basis and functional significance of scent production have also been investigated in *Petunia*. All wild accessions of *P. axillaris* produce a substantial amount of methylbenzoate [19••], a volatile that elicits a strong response from hawkmoths in electroantennogram assays [20], whereas the hummingbird-pollinated *P. exserta* produces no detectable volatile compounds at all. QTL mapping located two major loci underlying the scent production difference between *P. axillaris* and *P. exserta*: one on chromosome II and the other on chromosome VII [19••]. The latter locus contains a candidate gene *ODORANT1 (ODO1)*, an *R2R3-MYB* gene that belongs to a subgroup with *AtMYB42* and *AtMYB85* and has been shown to regulate benzenoid volatile production in *P. hybrida* cv Mitchell [21]. The level of *ODO1* transcripts is perfectly correlated with scent production in wild *Petunia* accessions, and is ~10-fold higher in scented *P. axillaris* than the background level in scentless *P. exserta* [19••]. Assaying the relative expression levels of *ODO1* alleles in the F1 hybrids indicated that the expression difference between the two species is because of